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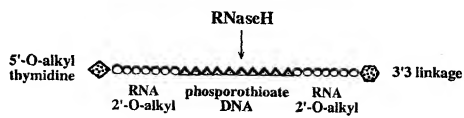
(54) Title: CHIMERIC ANTISENSE OLIGONUCLEOTIDES AND CELL TRANSFECTING FORMULATIONS THEREOF



(57) Abstract: Chimeric oligonucleotide of the formula 5'-W-X^1-Y-X^2-Z-3', where W represents a 5'-O-alkyl nucleotide, each of X^1 and X^2 represented a block of seven to twelve phosphodiester-linked 2'-O-alkyl ribonucleotides, Y represents a block of five to twelve phosphorothioate-linked deoxyribonucleotides, and Z represents a blocking group effective to block nuclease activity at the 3' end of the oligonucleotide, are described. These compounds exhibit high resistance to endo- and exonucleases, high sequence specificity, and the ability to activate RNAse H, as evidenced by efficient and long-lasting suppression of target mRNA. The oligonucleotides are preferably transfected into cells in formulations containing a lipid-peptoid conjugate carrier molecule of the formula L-linker-[$N(CH_2CH_2NH_2)CH_2(C{=}O)$-$N(CH_2CH_2R)CH_2(C{=}O)$-$N(CH_2CH_2R)CH_2(C{=}O)]_k$-$NH_2$, where L is a lipid moiety, including a steroid, and each group R is independently selected from alkyl, aminoalkyl, and aralkyl.

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Chimeric Antisense Oligonucleotides and Cell Transfecting Formulations Thereof

Field of the Invention

The present invention relates to antisense oligonucleotides, and more particularly to chimeric antisense oligonucleotides which exhibit high resistance to endo- and exonucleases, high sequence specificity, and the ability to activate RNAse H, as evidenced by efficient and long-lasting knockout of target mRNA. Also provided are formulations of the oligonucleotides with carrier molecules which provide efficient transfection into cells.

Background of the Invention

The use of antisense oligonucleotides to specifically inhibit the function of targeted genes has been the subject of extensive research, due to its promise in selective antiviral and anticancer therapy. Many studies have been directed to the design of oligonucleotide analogs having an optimal combination of properties, including stability (*i.e.* resistance to cellular nucleases), cellular uptake, DNA/RNA binding affinity and specificity, and efficiency of inhibition. Because the phosphodiester linkages of native nucleic acids are degraded by endo- and exonucleases, many early studies were directed to designing nuclease-resistant analogs. Phosphorothioates are one such class of compounds, which are relatively stable *in vivo* and retain the ability to activate RNAse H, the primary mechanism by which antisense oligonucleotides deactivate target RNA. However, the use of phosphorothioates presents several disadvantages, including a high level of non-specific binding to other cellular components, often leading to unwanted side effects, and reduced binding affinity for RNA.

Oligomeric ribonucleotides substituted at the 2'-oxygen show high RNA binding affinities and, in comparison to the unsubstituted ribonucleotides, reduced sensitivity to endogenous nucleases. Although 2'-O-methyl substituted ribonucleotides provide greater binding affinity than those having larger substituents (*e.g.* ethyl, propyl, pentyl, allyl), the larger substituents are reported to confer greater exonuclease resistance (see, for example, Monia *et al.*, *J. Biol. Chem.* **271**(24):14533, 1996). Arrow *et al.* (U.S. Patent No. 5,849,902) stated that "2'-O-methyl bases with phosphodiester linkages are degraded by exonucleases and so are not suitable for use

in cell or therapeutic applications of antisense." Phosphorothioate and phosphotriester linkages were recommended by the latter group as having greater stability, even though they presented the disadvantages of reduced binding affinity, more difficult synthesis (phosphotriester) and/or greater toxicity (phosphorothioate).

Therefore, there is still a need for antisense oligonucleotide compositions with optimal combinations of antisense activity, target binding affinity, biocompatibility, and stability.

Summary of the Invention

The present invention includes, in one aspect, a chimeric oligonucleotide having the formula 5'–W–X^1–Y–X^2–Z–3', where W represents a 5'-O-alkyl nucleotide, such as a 5'-O-alkyl thymidine; each of X^1 and X^2 represents a block of seven to twelve phosphodiester-linked 2'-O-alkyl ribonucleotides; Y represents a block of five to twelve phosphorothioate-linked deoxyribonucleotides; and Z represents a blocking group effective to block nuclease activity at the 3' end of the oligonucleotide. In one embodiment, Z is a 3-to-3' linked nucleotide. In further embodiments, the alkyl groups of the 5'-O-alkyl nucleotide and/or the 2'-O-alkyl ribonucleotides are methyl groups. In still further embodiments, groups W and/or Z are linked to X^1 and X^2, respectively, via phosphodiester linkages, phosphotriester, phosphorothioate, or phosphoramidate linkages. Preferably, W is linked via a phosphodiester or phosphorothioate linkage, and Z is linked via a relatively nuclease-resistant linkage; *i.e.* a phosphotriester, phosphorothioate, or phosphoramidate linkage.

In specific embodiments, the segment X^1–Y–X^2 of the chimeric oligonucleotide has a sequence represented by any of SEQ ID NOs: 1-24 disclosed herein.

In another aspect, the invention provides a therapeutic composition which comprises an oligonucleotide as described above in a pharmaceutically acceptable vehicle. In preferred embodiments, the vehicle includes a lipid-cationic peptoid conjugate or "lipitoid". One class of lipid-cationic peptoid conjugates includes compounds of the formula:

L-linker-$[N(CH_2CH_2NH_2)CH_2(C{=}O)\text{-}N(CH_2CH_2R)CH_2(C{=}O)\text{-}N(CH_2CH_2R)CH_2(C{=}O)]_1\text{-}NH_2$,

where the lipid group L is a fatty acid-derived group, such as a phospholipid group (*i.e.* $ROOCCH_2CH(COOR)CH_2OP(O)_2O$–), having fatty alkyl or alkenyl chains between about 8 and 24 carbon atoms in length, or a steroid-derived group, such as a

cholesteryl group, and the portion of the molecule to the right of the linker is the peptoid segment. In the peptoid segment, R is selected from alkyl (branched or unbranched), aminoalkyl, and aralkyl. As used herein, "aralkyl" refers to an alkyl, preferably lower alkyl, substituent which is further substituted with an aryl group; one example is a benzyl group. "Aryl" refers to a substituted or unsubstituted monovalent aromatic radical having a single ring (*e.g.*, benzene) or two condensed rings (*e.g.*, naphthyl). This term includes heteroaryl groups, which are aromatic ring groups having one or more nitrogen, oxygen, or sulfur atoms in the ring, such as furyl, pyrrole, pyridyl, and indole. By "substituted" is meant that one or more ring hydrogens in the aryl group is replaced with a substituent, preferably selected from a halide, a lower alkyl or lower alkoxy group, halomethyl, or haloethyl.

In specific embodiments, R is isopropyl or 4-methoxyphenyl. A single lipitoid may include different groups R, or they may be the same within the molecule.

The linker may be a direct bond, or it may be a substantially linear linking group, such as an oligopeptide or an alkyl chain, of any effective length. The linker may also be an alkyl chain having one or more heteroatom-containing linkages, selected from the group consisting of ester, amide, carbonate, carbamate, disulfide, peptide, and ether, at either terminus of the chain or intervening between alkyl bonds. In selected embodiments, the linker is from 2 to about 30 atoms, or from 3 to about 15 atoms, in length.

In another aspect, the invention provides a method of inhibiting expression of a target gene in a subject, which comprises administering to the subject, in a pharmaceutically acceptable vehicle, an amount of a chimeric oligonucleotide effective to specifically hybridize to all or part of a selected target nucleic acid sequence derived from the gene, where the chimeric oligonucleotide has a structure as described above. In one embodiment, the target nucleic acid sequence is a mRNA derived from the target gene. In specific embodiments, the segment X^1-Y-X^2 of the chimeric oligonucleotide has a sequence represented by any of SEQ ID NOs: 1-24 disclosed herein. In further embodiments, the vehicle includes a lipid-cationic peptoid conjugate such as described above.

As shown herein, the chimeric oligonucleotides of the invention provided surprisingly high stability and efficient and long-lasting knockout of target mRNA.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows a schematic representation of a chimeric oligonucleotide, in accordance with one embodiment of the invention;

Figure 2 shows a selection of phospholipid-peptoid conjugates ("lipitoids") and cholesterol-peptoid conjugates ("cholesteroids") useful as oligonucleotide carriers in compositions and methods of the invention;

Figure 3 shows the effect on AKT1 mRNA level of antisense oligos to AKT1 delivered to HT1080 cells via Effectene™, Lipitoid 1, and peptoid 1, and control oligos (AKT2-AS, AKT2-RC, and AKT1-RC) delivered via Effectene™;

Figure 4 shows the effect on AKT1 mRNA level of antisense oligos to AKT1 delivered to colon cancer cells (Lovo) in conjunction with: Lipitoid 1, two different charge ratios of Lipitoid 2 (DMPE(NaeNiaNia)$_3$), two different charge ratios of Cholesteroid 1 (Chol-β-ala-(NaeNmpeNmpe)$_3$), and the commercially available transfection agent Cytofectin™;

Figures 5-7 show the effects on cell proliferation of transfection of Lovo, Km12L4, and Colo320DM colon cancer cells, respectively, with chimeric oligonucleotides of the invention, in conjunction with different lipitoid and cholesteroid carriers; and

Figure 8 shows the results of several cell viability assays on Km12L4 and HCT-166 cells transfected with oligonucleotides in conjugation with lipitoids 1 and 2, cholesteroids 1 and 3, and the commercially available transfection agents Lipofectin® and Cytofectin™, where the white regions indicate levels of healthy cells.

Detailed Description of the Invention

I. Chimeric Antisense Oligonucleotides

A. Structure

The chimeric oligonucleotides of the invention have the general structure shown below:

$$5'\text{-}W\text{-}X^1\text{-}Y\text{-}X^2\text{-}Z\text{-}3'$$

In this structure, the central portion of the molecule, represented by Y, is a block of between five and twelve phosphorothioate-linked deoxyribonucleotides (phosphorothioate DNA, or PS DNA). In one embodiment, the block Y is effective to activate RNAse H when hybridized to a sufficiently complementary strand of RNA, thus promoting cleavage of the RNA. Block Y is flanked by two binding blocks, represented by X^1 and X^2, each having between seven and twelve phosphodiester-linked 2'-O-alkyl ribonucleotide subunits (phosphodiester 2'-O alkyl RNA, or PO 2'-O-alkyl RNA). As used herein, "alkyl" refers to a fully saturated acyclic monovalent radical containing carbon and hydrogen, which may be branched or a straight chain; examples of alkyl groups are methyl, ethyl, n-butyl, t-butyl, n-heptyl, and isopropyl. "Lower alkyl" refers to an alkyl radical of one to six carbon atoms, and preferably one to four carbon atoms.

The alkyl groups of the 2'-O-alkyl ribonucleotide subunits are preferably lower alkyl groups. In one embodiment, the alkyl groups are methyl groups, which provide generally superior binding and cellular uptake in comparison to longer alkyl groups. The binding blocks, while not necessarily effective to participate in activation of RNAse H, provide binding affinity to sufficiently complementary RNA strands and may also provide reduced cellular toxicity compared to phosphorothioate-linked subunits.

Blocking groups Z and W are provided at the 3' and 5' termini, respectively. In one embodiment, the groups W and Z are linked to the respective X blocks by phosphodiester linkages; in another embodiment, they are attached via phosphorothioate linkages. The 3'-blocking group Z is preferably a 3'-to-3' linked nucleotide, although this terminus may also be blocked by other methods, *e.g.* by attachment of the terminal nucleotide via a relatively nuclease-stable linkage (*e.g.* phosphorothioate, phosphoramidate, phosphotriester) or appendage of a non-nucleotide moiety.

The 5'-terminus is blocked with a 5'-O-alkyl nucleotide subunit (W), where alkyl is preferably lower alkyl. In one embodiment, W is a 5'-O-methyl thymidine. This blocking group is found to confer stability to the chimeric oligonucleotides in cell culture and in serum. For example, the duration of mRNA knockout in cell cultures (discussed further below) typically ranged from 3-5 days post transfection. In addition to providing stability, this blocking group, and the 3'-to-3' nucleotide

blocking group, were found not to interfere with uptake or distribution of the oligonucleotides.

The chimeric oligonucleotides of the invention can be prepared using solution phase or, preferably, solid phase synthesis, according to established procedures. Synthesis of an exemplary chimeric oligonucleotide, such as shown in Fig. 1, is described in Example 1.

B. Antisense Activity

Antisense chimeric oligonucleotides based on the formula above, having sequences directed against AKT1 (SEQ ID NO: 1) or AKT2 (SEQ ID NO: 2), were prepared as described in Example 1. The oligonucleotides also included a 5'-terminal 5'-O-methyl thymidine, as indicated by the formula above. In these oligonucleotides, X^1 and X^2 were seven-base blocks of 2'-O-methyl PO RNA, Y is PS DNA, Z was a 3'-to-3' linked nucleotide, and W was a 5'-O-methyl thymidine. Both Z and W were linked to the respective X blocks via phosphodiester linkages.

When transfected into cells as described in Example 2, chimeric antisense oligonucleotides of the invention having various sequences (see Table 1) showed surprisingly effective degradation of endogenous mRNA, resulting in a loss of activity of the respective genes. Figs. 5 and 6 show levels of endogenous AKT1 mRNA in colon cancer cells and HT1080 cells, respectively, transfected with anti-AKT1 chimeric oligonucleotides (SEQ ID NO: 1). Similarly, a chimeric antisense oligonucleotide directed against hCHK1 (SEQ ID NO: 3) showed degradation of endogenous mRNA, loss of chk1 kinase activity, and loss of chk1 function (*i.e.* G2 cell cycle checkpoint control).

Additional chimeric oligonucleotides having the sequences shown in Table 1 were prepared and administered to cells as described in Example 2. (Each oligonucleotide included a 5'-O-methyl thymidine, as described above, which is not shown in the listed sequences.) In these oligonucleotides, with reference to the formula above, X^1 and X^2 are seven-base blocks of 2'-O-methyl PO RNA, Y is a nine- to eleven-base block of PS DNA, Z is a 3'-to-3' linked nucleotide, and W is a 5'-O-methyl thymidine. Both Z and W are linked via phosphodiester linkages.

With the exception of the mTyr oligo (SEQ ID NO: 17-18), which was transfected into B16 melanoma cells, all oligos shown in Table 1 were transfected into HT1080 cells, using "Lipitoid 1" (see below) for transfection, and incubated for

24 hours. The table gives the approximate level of mRNA knockout observed in each case. Reduction in mRNA levels of 90% or more were frequently observed, as shown in the Table.

Table 1

SEQ ID NO:	Antisense Oligonucleotide Sequence (X^1–Y–X^2 Segment)	Gene	Nucleotide Position in Gene	Locus/Acc for Genbank	mRNA knockout
1	CCATAGTGAGGTTGCATCTGGTGCC	AKT1	AKT1-2074	NM005163	>90%
2	GTTCCCTTGCCAAGGAGTTTGAGAT	AKT2	AKT2-548	NM001626	>85%
3	CCCAGAGCCGATGGTCCGATCATGT	CHK1	CHK1-1460	CHEK1	>90%
4	GACCCACTTCCCTGAAAATCCGAAA	CHK2	CHK2-430	AF086904	>90%
5	CGGCGTTTTCCTTTCCCTACAAGC		CHK2-518	AF086904	
6	AGCGGCAGAAGTTGAGGTATGTTGA	CK1E	CK1E-766	HUMCSNK1E	>80%
7	CCTGCCAGTATGAAGTTGGGAAGCG	E1AF	E1AF-1729	HUME1AF	>90%
8	GCGAAGTCCGTCTGTTCCTGTTTGA		E1AF-710	HUME1AF	
9	TCTTCCTCACAGACCTTCGGGCAAG	IGFR1	IGF1R-1025	HSIGFIRR	>80%
10	TGCTGATAGTCGTTGCGGATGTCG		IGF1R-156	HSIGFIRR	
11	GTGTTTGTTCAGGGTTCCATTTCGG	ILK	ILK-687	HSU40282	>90%
12	GCATGTGGAAGGTAGGGAGGCAAGA	KRAS	KRAS-2576	HUMKRASM	>85%
13	ACCATATACCCAGTGCCTTGTGCGG		KRAS-3352	HUMKRASM	
14	GAAGCCCCACTTGCGGTCGTCAT	MMP2	MMP2-1098	HUMCN4GEL	>80%
15	ACGAGCAAAGGCATCATCCACTGTC		MMP2-367	HUMCN4GEL	
16	GCTTTCTCTCGGTACTGGAAGACGT	MMP9	MMP9-2007	HUM4COLA	>80%
17	AACCCATGAAGTTGCCTGAGCACTG	mTyr	MTYR-332	MUSTYR	>90%
18	TTTCAGGGTGACGACCTCCCAAGTA		MTYR-814	MUSTYR	
19	ATCTGGTCGCCTCATTTGCTCAACT	p110α	P110A-2205	HSU79143	>95%
20	TTTCTTCACGGTTGCCTACTGGTTC		P110A-307	HSU79143	
21	TGATGAAGAGATTCCCATGCCGTCG	p110β	P110B-2980	S67334	>90%
22	TGTAGTCTTTCCGAACTGTGTGGGC		P110B-3181	S67334	
23	CTGTGAGCAACAGCTGTCGTCGTCT	PDK1	PDK1-1494	NM002613	>90%
24	GGCAGTCATTAGCAGGGTGATGGTG	UPAR	UPAR-1242	HSU08839	>80%

II. Transfection Agents

A variety of strategies exist for delivery of nucleic acid compositions to cells. Viral vectors provide relatively efficient delivery, but in some cases present safety problems due to the risk of immunological complications or unwanted propagation in the subject. Adenoviral vectors have shown certain advantages in that they do not integrate into the genome of the cell and can be transduced into resting cells. However, all of these vectors must be prepared by time consuming recombinant DNA techniques. Oligonucleotides may also be delivered to cells via chemical transfection agents, which have been the subject of much recent work. These agents include polycationic molecules, such as polylysine, and cationic lipids. The liposomal composition Lipofectin® (Felgner *et al., PNAS* **84**:7413, 1987), containing the cationic lipid DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) and the neutral phospholipid DOPE (dioleyl phosphatidyl ethanolamine), is

widely used. Any of these methods, as well as other methods such as calcium phosphate mediated transfection, can be used to deliver the oligonucleotides of the invention, according to reported procedures.

One method of delivery involves the use of transfection agents known as "lipitoids" and "cholesteroids", described, for example, in co-owned PCT publications WO 98/06437 and WO 99/08711 (Zuckermann *et al.*), based on US serial numbers 60/023,867, 60/054,743, and 09/132,808, which are hereby incorporated by reference. These lipid-cationic peptoid conjugates are shown in these references to be effective reagents for the delivery of plasmid DNA to cells *in vitro*. It is shown herein that such compounds efficiently deliver oligonucleotides into a variety of primary and tumor cell lines. The efficiency of delivery was assessed by fluorescence analysis of FITC-labeled oligonucleotides, or by monitoring mRNA levels after transfection of chimeric antisense oligonucleotides, as described further below.

Any of the carriers described in the above-referenced applications are suitable for use in transfection of the oligonucleotides described herein. Further disclosure of steroids useful for incorporating into steroid-cationic peptoid conjugates is found in PCT publication WO 97/46223 (Fasbender *et al.*) and corresponding U.S. Patent No. 5,935,936, which are hereby incorporated by reference.

These compounds may be prepared by conventional solution or solid-phase synthesis. In one such procedure, as described in Zuckermann *et al.*, cited above, the N-terminus of a resin-bound peptoid is acylated with a spacer such as Fmoc-aminohexanoic acid or Fmoc-β-alanine. After removal of the Fmoc group, the primary amino group is reacted with cholesterol chloroformate to form a carbamate linkage, *e.g.* as shown in Cholesteroids 2, 3, and 4 of Fig. 2. The product is then cleaved from the resin with trifluoroacetic acid and purified by reverse-phase HPLC. A fatty acid-derived lipid moiety, such as a phospholipid, may be used in place of the steroid moiety, as also shown in Fig. 2.

The steroid or other lipid moiety may also be linked to the peptoid moiety by other linkages, of any effective length, readily available to the skilled practitioner. The linker is a chain up to about 30 bonds in length, and more preferably up to about 15 bonds in length, though any effective length may be used. The chain is typically linear or substantially linear, although branched chains (including oligopeptides) and

linkers containing intervening cyclic groups can also be used. The linker generally comprises alkyl (C-C) bonds and one or more functional groups such as ester, amide, carbonate, carbamate, disulfide, peptide or ether bonds. The linker may comprise multiple functional groups, as in a succinate ester or polyether, or it may be an oligopeptide, preferably a 2- to 10-mer, and more preferably a 2- to 5-mer. The steroid or lipid moiety and peptoid segment can also be joined by a direct bond.

In certain embodiments, the linker incorporates one or more bonds which are susceptible to cleavage under appropriate conditions *in vivo*; for example, hydrolyzable ester, carbonate, carbamate, or peptide bonds; disulfide bonds, which are cleavable in cellular compartments having a sufficiently reducing environment; and peptide bonds, cleavable by endogenous peptidases. With respect to the latter, polypeptide linkers having ten or fewer, or, in further embodiments, five or fewer peptide linkages are contemplated, though longer linkers may also be used.

In particular embodiments, the lipid-cationic peptoid conjugate belongs to a class of compounds having the formula:

$L\text{-}(CH_2)_n\text{-}(C{=}O)\text{-}[N(CH_2CH_2NH_2)CH_2(C{=}O)\text{-}N(CH_2CH_2R)CH_2(C{=}O)\text{-}N(CH_2CH_2R)CH_2(C{=}O)]_3\text{-}NH_2$,

where L is selected from (i) a phosphatidylethanolamino group (*i.e.* $ROOCCH_2CH(COOR)CH_2OP(O)_2O\text{–}CH_2CH_2NH_2\text{–}$), having fatty alkyl or alkenyl chains between about 8 and 24 carbon atoms in length, and (ii) a cholesteryl group linked to the adjacent $\text{-}(CH_2)_n\text{-}$ segment by an ester, amide or carbamate linkage; n is 1-5; and R is selected from isopropyl and 4-methoxyphenyl. Representative structures of this class, shown in Fig. 2, are given the following designations herein:

Lipitoid 1, or L1	$DMPE(NacNmpeNmpe)_3$
Lipitoid 2, or L2	$DMPE(NacNiaNia)_3$
Cholesteroid 1, or Chol 1	$Chol\text{-}\beta\text{-}ala\text{-}(NacNmpeNmpe)_3$
Cholesteroid 2, or Chol 2	$Chol\text{-}Ahx\text{-}(NacNmpeNmpe)_3$
Cholesteroid 3, or Chol 3	$Chol\text{-}\beta\text{-}ala\text{-}(NacNiaNia)_3$
Cholesteroid 4, or Chol 4	$Chol\text{-}Ahx\text{-}(NacNiaNia)_3$

As used herein, the term "lipitoid" may be used generically to include both lipitoids and cholesteroids, unless referring to a particular Lipitoid, such as L1 or L2, above.

To prepare transfecting compositions, an aqueous solution of a peptoid, lipitoid

or cholesteroid is formulated with the oligonucleotide, as described in Example 2A. The components are preferably used in relative amounts such that there are at least two, and preferably two to four, positive lipitoid charges for every DNA negative charge. The exact ratio of antisense oligonucleotide to lipitoid is preferably determined empirically for each cell type, but is generally in the range of 1.5-2 nmol lipitoid /μg antisense oligonucleotide. Cells may be transfected as described above and in Example 2B.

The extent of delivery of FITC-labeled chimeric oligonucleotides into human fibrosarcoma (HT1080) cells was assessed via fluorescence analysis. (All oligonucleotides used in the subsequent studies were chimeric oligonucleotides as described for the studies represented in Table 1.) The sequence of the oligonucleotides was the reverse control of PDK1 (SEQ ID NO: 25, the reverse of SEQ ID NO: 23) so that the effect of the oligonucleotides on the cells would be minimal. The oligonucleotides were transfected via complexation with (a) a commercially available transfection agent, Effectene™, (b) the peptoid $(NaeNmpeNmpe)_3$ (peptoid 1), (c) Lipitoid 1, in a 1:4 charge ratio of oligo to lipitoid, and (d) Lipitoid 1, in a 1:3 charge ratio. In comparison to Effectene™, Lipitoid 1 gave a significantly higher transfection efficiency and higher degree of nuclear delivery of the oligonucleotide, as evidenced by fluorescence analysis of the transfected cells. The higher lipitoid/oligo charge ratio (1:4; c) also appeared to be more effective than the 1:3 ratio.

Figure 3 shows the reduction in endogenous AKT1 mRNA in HT1080 cells resulting from transfection of a chimeric antisense oligonucleotide to AKT1, as described above (AKT1-AS), in comparison to control oligonucleotides AKT1-RC (RC=reverse control; SEQ ID NO: 26; reverse of SEQ ID NO: 1), AKT2-AS, and AKT2-RC (SEQ ID NO: 27; reverse of SEQ ID NO: 2). The same oligos were also delivered by commercial lipids (Effectene™) and peptoids ($(NaeNmpeNmpe)_3$). The results, depicted in Fig. 3, show that L1-transfected AKT1 chimeric antisense oligonucleotides gave the most pronounced reduction in the target mRNA level.

Efficiency of oligonucleotide delivery by cholesteroids has been found to be similar or superior to that of (non-steroid) lipitoids. For example, as shown in Fig. 4, delivery of the chimeric anti-AKT1 oligo described above by Cholesteroid 1

achieved a better AKT1 mRNA knockout than delivery by Lipitoid 1 or Lipitoid 2 in a colon cancer cell line (Lovo).

The cholesteroids provide the additional benefit of substantially reduced toxicity to cells *in vitro*. Fig. 5 shows a 4 day proliferation assay, conducted as described in Example 3, of Lovo colon cancer cells following transfection of 50-300 nM of oligonucleotides. (Again, reverse control PDK1 chimeric oligonucleotides, expected to be non-active, were used.). These charts demonstrate the significant increase in proliferation and viability of the Lovo cells following an oligonucleotide transfection with Cholesteroids 2 and 3 (Fig. 5B,D) as compared with transfection with Lipitoids 1 and 2 (Fig. 5A,C). This effect is not limited to this cell type, and was also observed in proliferation assays of Km12L4 colon cancer cells (Fig. 6) and Colo320DM colon cancer cells (Fig. 7).

To further investigate the reduced toxicity of the cholesteroids, a FACS analysis of cells was carried out, following transfection (see Example 4), to determine the number of necrotic (PI+), early apoptotic (annexin+), late apoptotic (annexin+ / PI+) and healthy cells (annexin- / PI-). The white columns in Fig. 8 reflect the number of healthy cells, while colored portions of the bars (demarcated by short line segments for clarity) represent dead or dying cells. The analysis was performed on Km12L4 (Fig. 8A-B) and HCT116 cells (Fig. 8C-D). The percentage of dying cells was determined 4 hours (Fig. 8A,C) or 24 hours (Fig. 8B,D) post-transfection. While different cell types show different sensitivity to the transfection, cells transfected with cholesteroids consistently contained the most healthy cells and showed the lowest degree of cell death. This lower toxicity was also seen in comparison of cholesteroids with the commercially available lipids Lipofectamine® and Cytofectin™.

EXAMPLES

The following examples illustrate but are not intended in any way to limit the invention.

Example 1. Synthesis of Chimeric poRNA-psDNA-poRNA Oligonucleotides

The chimeric oligonucleotides were prepared using solid phase synthesis, according to established procedures. A PerSpective Biosystems (Framingham, MA)

8909 Synthesizer and an ABI 394 Synthesizer (ABI/Perkin-Elmer, Foster City, CA) were used for the RNA additions and the phosphorothioate linked DNA additions, respectively. It is also possible to perform the synthesis using only one instrument with eight amidite reagent bottles. Unless otherwise noted, all reagent preparation and synthesis was performed using the manufacturers' standard protocols.

The 5'-CPG support column, 5'-O-methyl-RNA phosphoramidites, 5'-O-methyl-dT-CE phosphoramidite, and sulfurizing reagent, 3H-1,2-benzodithiole-3-one-2,2-dioxide, were all obtained from Glen Research (Sterling, VA).

To carry out a representative synthesis, the last seven bases of the desired sequence were entered into the 8909 Synthesizer, supplied with 2'-O-methyl-RNA phosphoramidites, the appropriate 5'-CPG column was attached, and a 1 μmole-scale RNA synthesis was performed with the final DMT on.

The column was then removed from the 8909 and attached to the ABI 394. The sulfurizing agent was installed in position 15 (to replace the oxidizer), and a synthesis for the phosphorothioate middle section of the oligo was carried out, using the 1 μmole Sulfur program with the final DMT on.

The column was then removed and replaced on the 8909. The last seven 2'-O-methyl RNA bases were added, using the 1 μmole RNA program, DMT on. Finally, the chain terminator, 5'-O-methyl-dT-CE (cyanoethyl) phosphoramidite, was added, using a 1 μmole DNA protocol modified to extend the coupling time to 300 seconds. The oligonucleotide was cleaved from the support, deprotected and gel purified using standard methods.

Example 2. Antisense Inhibition of Target RNA

A. Preparation of Transfection Mixture

For each transfection mixture, a carrier molecule, preferably a lipitoid or cholesteroid, was prepared to a working concentration of 0.5 mM in water, sonicated to yield a uniform solution, and filtered through a 0.45 μm PVDF membrane. The antisense oligonucleotide was prepared to a working concentration of 100 μM in sterile Millipore water.

The oligonucleotide was diluted in OptiMEM™ (Gibco/BRL), in a microfuge tube, to 2 μM, or approximately 20 μg oligo/ml of OptiMEM™. In a separate microfuge tube, lipitoid or cholesteroid, typically in the amount of about 1.5-2 nmol

lipitoid/μg antisense oligonucleotide, was diluted into the same volume of OptiMEM™ used to dilute the oligonucleotide. The diluted antisense oligonucleotide was immediately added to the diluted lipitoid and mixed by pipetting up and down.

B. Transfection

Cells were plated on tissue culture dishes one day in advance of transfection, in growth media with serum, to yield a density at transfection of 60-90%. The oligonucleotide/lipitoid mixture was added to the cells, immediately after mixing, to a final concentration of 100-300 nM antisense oligonucleotide. Cells were incubated with the transfection mixture at 37°C, 5% CO_2 for 4-24 hours. After incubation, the transfection mixture was removed and replaced with normal growth media with serum.

Total RNA was extracted using the RNeasy™ kit (Quiagen Corporation, Chatsworth, CA), according to manufacturer's protocols.

C. Reverse Transcription

The level of target mRNA was quantitated using the Roche LightCycler™ real-time PCR machine. Values for the target mRNA were normalized versus an internal control (*e.g.* beta-actin).

For each 20 μl reaction, extracted RNA (generally 0.2-1 μg total) was placed into a sterile 0.5 or 1.5 ml microcentrifuge tube, and water was added to a total volume of 12.5 μl. To each tube was added 7.5 μl of a buffer/enzyme mixture, prepared by mixing (in the order listed) 2.5 μl H_2O, 2.0 μl 10X reaction buffer, 10 μl oligo dT (20 pmol), 1.0 μl dNTP mix (10 mM each), 0.5 μl RNAsin® (20u) (Ambion, Inc., Hialeah, FL), and 0.5 μl MMLV reverse transcriptase (50u) (Ambion, Inc.). The contents were mixed by pipetting up and down, and the reaction mixture was incubated at 42°C for 1 hour. The contents of each tube were centrifuged prior to amplification.

D. LightCycler™ Amplification of RT Reactions

An amplification mixture was prepared by mixing in the following order: 1X PCR buffer II, 3 mM $MgCl_2$, 140 μM each dNTP, 0.175 pmol each oligo, 1:50,000 dil of SYBR® Green, 0.25 mg/ml BSA, 1 unit *Taq* polymerase, and H_2O to 20 μl. (PCR buffer II is available in 10X concentration from Perkin-Elmer (Norwalk, CT). In 1X concentration it contains 10 mM Tris pH 8.3 and 50 mM KCl. SYBR® Green

(Molecular Probes, Eugene, OR) is a dye which fluoresces when bound to double stranded DNA. As double stranded PCR product is produced during amplification, the fluorescence from SYBR® Green increases.)

To each 20 µl aliquot of amplification mixture, 2 µl of template RT was added, and amplification was carried out according to standard protocols.

Example 3. Cell Proliferation Assay

Cells were seeded into 96 well plates at a density of 5000 cells per well. For a 4 day proliferation assay, 5 independent 96 well plates were prepared, one for each day. After overnight incubation, cells were transfected using the procedure described above. On each day of the proliferation assay, all medium was removed from one plate and frozen at -70°C. On day four, all plates were developed with the Quantos™ assay kit (Stratagene, La Jolla, CA) which determines the amount of DNA, and thus the number of cells, in each well.

Example 4. Cytotoxicity Assay

Cells were seeded in 35 mm dishes at 35000 cells/well and allowed to attach overnight. Cells were then transfected with oligonucleotide/lipid formulations at 50-300 nM and incubated for 4 or 24 hours. Cells were harvested 12 hours later, including the medium containing floating cells. Live cells were then stained with propidium iodine (PI) to detect necrotic and apoptotic cells and counterstained with FITC-coupled Annexin V (which detects early and late apoptotic cells) according to the R&D Systems (Minneapolis, MN) Apoptosis Detection Kit instructions. The cells were then analyzed by FACS analysis to determine the relative number of PI+, annexin V+, PI+ annexin V+ and PI- / annexin V- cells. The results are expressed as percent (Fig. 8).

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications may be made without departing from the invention.

IT IS CLAIMED:

1. A chimeric oligonucleotide having the formula 5'–W–X^1–Y–X^2–Z–3', where W represents a 5'-O-alkyl nucleotide;

each of X^1 and X^2 represents a block of seven to twelve phosphodiester-linked 2'-O-alkyl ribonucleotides;

Y represents a block of five to twelve phosphorothioate-linked deoxyribonucleotides; and

Z represents a blocking group effective to block nuclease activity at the 3' end of the oligonucleotide.

2. The oligonucleotide of claim 1, wherein the alkyl groups of the 5'-O-alkyl nucleotide and the 2'-O-alkyl ribonucleotides are lower alkyl groups.

3. The oligonucleotide of claim 2, wherein the alkyl groups of the 2'-O-alkyl ribonucleotides are methyl groups.

4. The oligonucleotide of claim 1, wherein the 5'-O-alkyl nucleotide is a 5'-O-alkyl thymidine.

5. The oligonucleotide of claim 1, wherein the 5'-O-alkyl nucleotide is linked to X^1 via a phosphodiester linkage or a phosphorothioate linkage.

6. The oligonucleotide of claim 1, wherein group Z is linked to X^2 via a linkage selected from the group consisting of a phosphotriester linkage, a phosphorothioate linkage, and a phosphoramidate linkage.

7. The oligonucleotide of claim 1, wherein Z is a 3-to-3' linked nucleotide.

8. The oligonucleotide of claim 1, wherein the segment X^1–Y–X^2 has a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1-24.

9. A composition useful for inhibiting expression of a target gene in a subject, comprising a chimeric oligonucleotide as recited in claim 1 in a pharmaceutically acceptable vehicle.

10. The composition of claim 9, wherein the vehicle includes a lipid-cationic peptoid conjugate of the formula:

L–linker–[$N(CH_2CH_2NH_2)CH_2(C=O)$-$N(CH_2CH_2R)CH_2(C=O)$-$N(CH_2CH_2R)CH_2(C=O)]_3$-NH_2

where

L is selected from a lipid moiety comprising at least one fatty alkyl or alkenyl chain between about 8 and 24 carbon atoms in length and a steroid;

each group R is independently selected from alkyl, aminoalkyl, and aralkyl, and

the linker is selected from the group consisting of a direct bond, an oligopeptide, a substantially linear alkyl chain from 2 to about 30 bonds in length, and a substantially linear chain from 2 to about 30 bonds in length consisting of alkyl bonds and one or more linkages selected from the group consisting of ester, amide, carbonate, carbamate, disulfide, peptide, and ether.

11. The composition of claim 10, wherein the linker is from 3 to about 15 bonds in length.

12. The composition of claim 10, wherein said fatty alkyl or alkenyl chain is between about 14 and 24 carbon atoms in length.

13. The composition of claim 10, wherein L is a phospholipid group, having two fatty alkyl or alkenyl chains between about 8 and 24 carbon atoms in length.

14. The composition of claim 10, wherein L is a cholesteryl group.

15. The composition of claim 10, wherein R is isopropyl or 4-methoxyphenyl.

16. The composition of claim 10, wherein the lipid-cationic peptoid conjugate is of the formula:

L–$(CH_2)_n$-(C=O)-[$N(CH_2CH_2NH_2)CH_2(C=O)$-$N(CH_2CH_2R)CH_2(C=O)$-$N(CH_2CH_2R)CH_2(C=O)]_3$-NH_2 ,

where

L is selected from (i) a phosphatidylethanolamino group, having fatty alkyl or alkenyl chains between about 8 and 24 carbon atoms in length, and (ii) a cholesteryl group linked to the adjacent -$(CH_2)_n$- segment by an ester, amide or carbamate linkage;

n is 1-5; and

R is selected from isopropyl and 4-methoxyphenyl.

17. The composition of claim 16, wherein the lipid-cationic peptoid conjugate is selected from the group consisting of compounds represented herein as:

(a) Lipitoid 1, or DMPE(NaeNmpeNmpe)$_3$;

(b) Lipitoid 2, DMPE(NaeNiaNia)$_3$;

(c) Cholesteroid 1, or Chol-β-ala-(NaeNmpeNmpe)$_3$;

(d) Cholesteroid 2, or Chol-Ahx-(NaeNmpeNmpe)$_3$;

(e) Cholesteroid 3, or Chol-β-ala-(NaeNiaNia)$_3$; and

(f) Cholesteroid 4, or Chol-Ahx-(NaeNiaNia)$_3$.

18. A method of inhibiting expression of a target gene in a subject, comprising administering to the subject, in a pharmaceutically acceptable vehicle, an amount of a chimeric oligonucleotide as recited in claim 1 which is effective to specifically hybridize to all or part of a selected target nucleic acid sequence derived from the gene.

19. The method of claim 18, wherein the target nucleic acid sequence is a mRNA derived from the target gene.

20. The method of claim 19, wherein the segment X^1–Y–X^2 of the chimeric oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1-24.

21. The method of claim 18, wherein the vehicle includes a lipid-cationic peptoid conjugate as recited in claim 11.

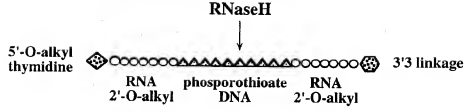
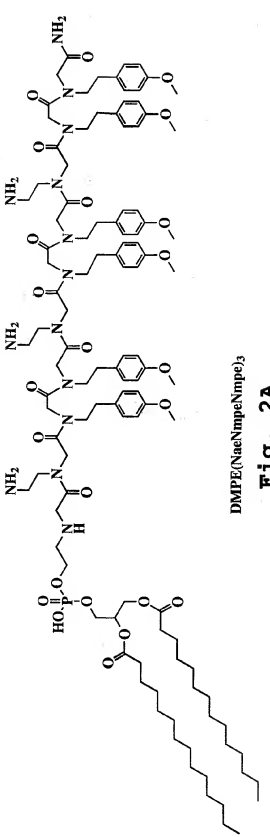
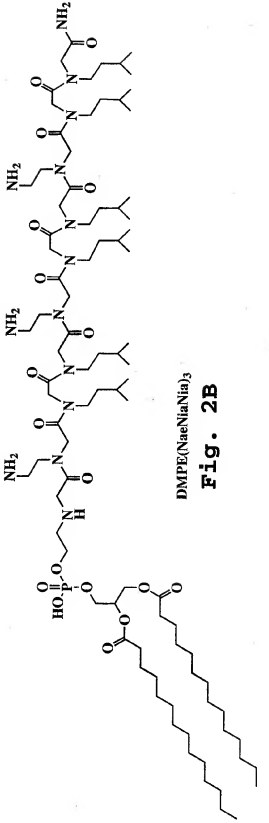


Fig. 1



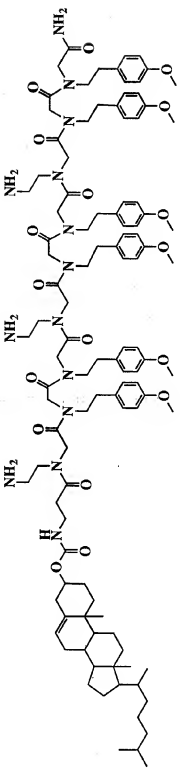
DMPE(NaeNmpeNmpe)$_3$

Fig. 2A



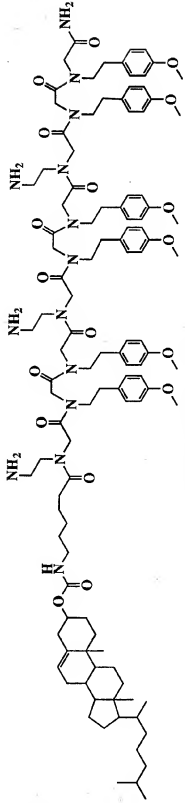
DMPE(NaeNiaNia)$_3$

Fig. 2B



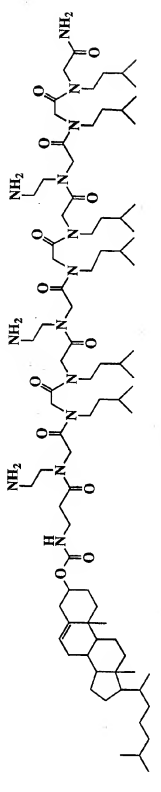
Chol-β-ala-(NaeNmpeNmpe)$_3$

Fig. 2C



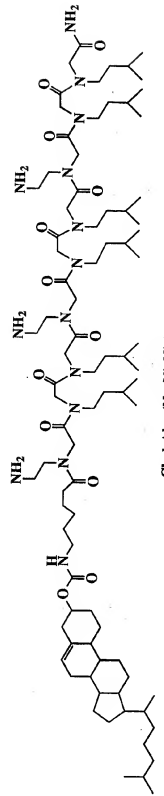
Chol-Alx-(NaeNmpeNmpe)$_3$

Fig. 2D



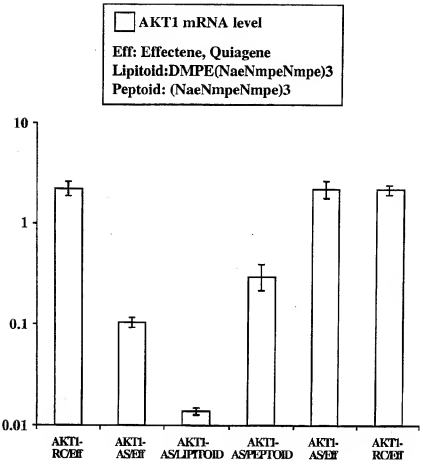
Chol-β-ala(NaeNiaNia)$_3$

Fig. 2E



Chol-Ahx-(NaeNiaNia)$_3$

Fig. 2F



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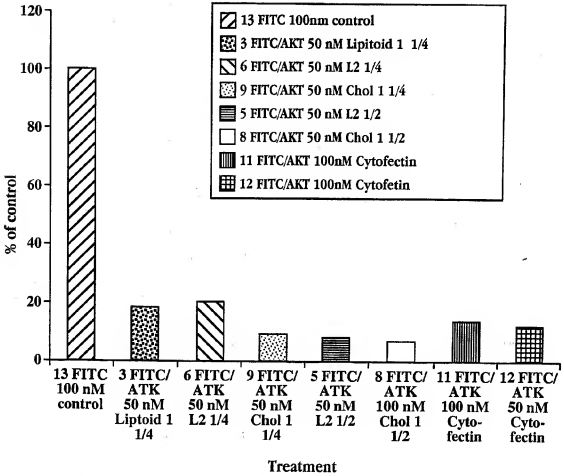

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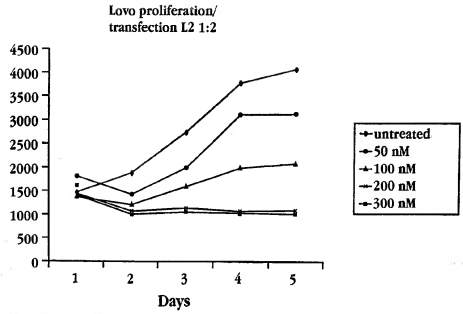

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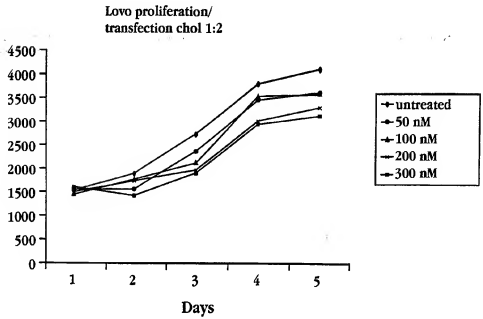

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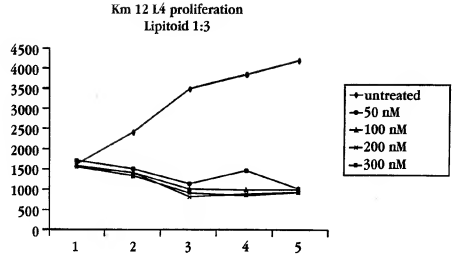

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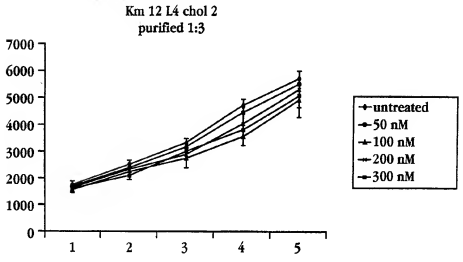

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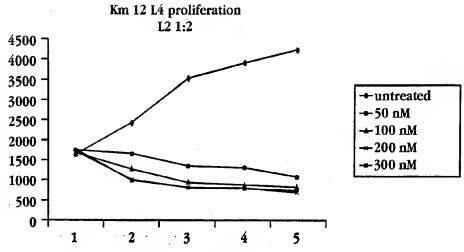
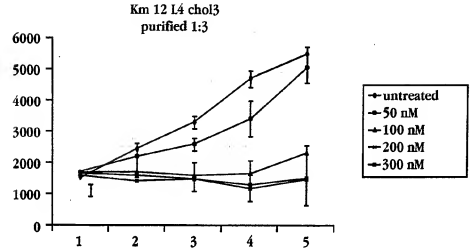


Fig. 6C



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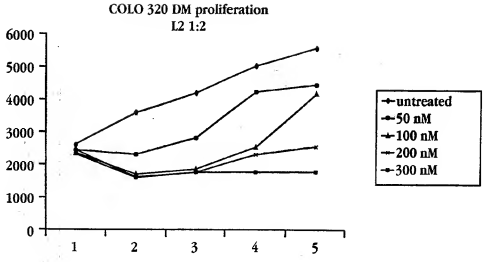

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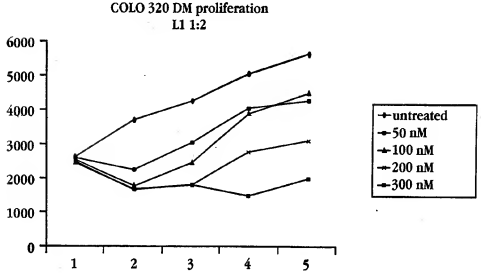

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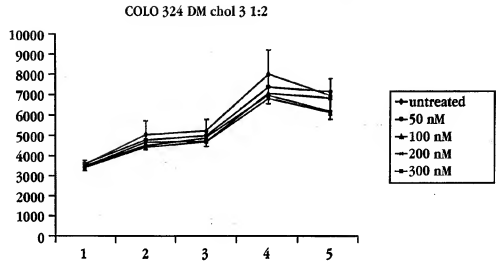

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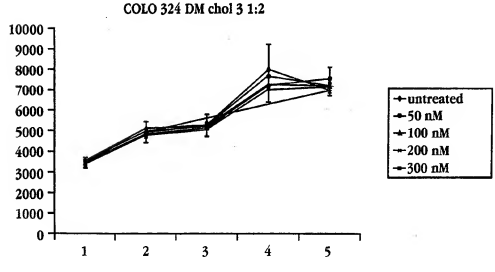

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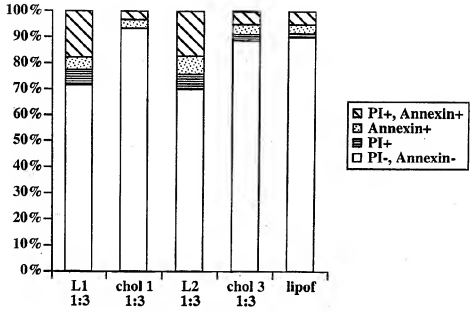

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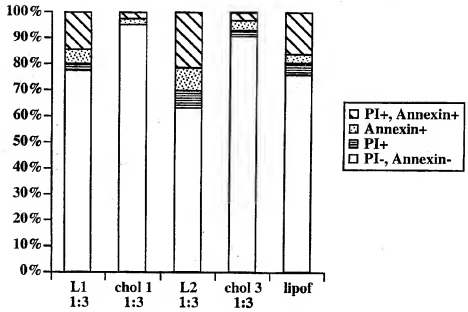

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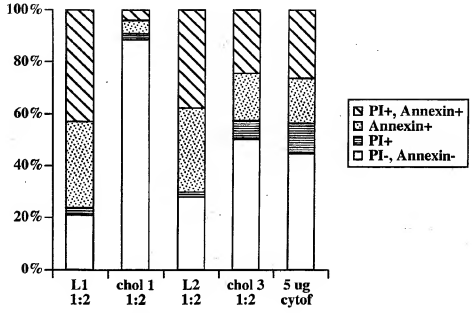

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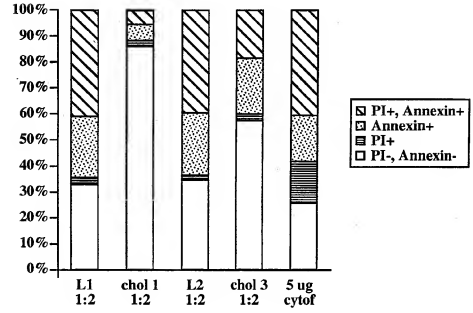

Fig. 8D

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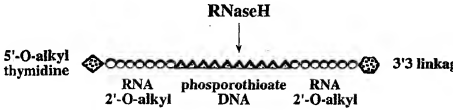
Published:
— *With international search report.*

(88) Date of publication of the international search report:
10 May 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CHIMERIC ANTISENSE OLIGONUCLEOTIDES AND CELL TRANSFECTING FORMULATIONS THEREOF



(57) Abstract: Chimeric oligonucleotide of the formula 5'-W-X¹-Y-X²-Z-3', where W represents a 5'-O-alkyl nucleotide, each of X¹ and X² represented a block of seven to twelve phosphodiester-linked 2'-O-alkyl ribonucleotides, Y represents a block of five to twelve phosphorothioate-linked deoxyribonucleotides, and Z represents a blocking group effective to block nuclease activity at the 3' end of the oligonucleotide, are described. These compounds exhibit high resistance to endo- and exonucleases, high sequence specificity, and the ability to activate RNAse H, as evidenced by efficient and long-lasting suppression of target mRNA. The oligonucleotides are preferably transfected into cells in formulations containing a lipid-peptoid conjugate carrier molecule of the formula L-linker-[$N(CH_2CH_2NH_2)CH_2(C{=}O)$-$N(CH_2CH_2R)CH_2(C{=}O)$-$N(CH_2CH_2R)CH_2(C{=}O)]_3$-$NH_2$, where L is a lipid moiety, including a steroid, and each group R is independently selected from alkyl, aminoalkyl, and aralkyl.

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/23290

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 A61K47/48 A61K31/7125 C07H21/00
//C12N15/88,A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STEIN ET AL.: "Applied Antisense Oligonucleotide Technology" 1998 , WILEY.LISS. INC. XP002119324 ALTAMANN ET AL. Chapter 4 page 73 -page 107; figure 4.8 ---	1-21
X	US 5 849 902 A (ARROW AMY ET AL) 15 December 1998 (1998-12-15)	1-7,9-21
A	column 3, line 43-63 -column 4, line 28-36; claims 1-21 ---	8
X	DE 197 50 702 A (HOECHST MARION ROUSSEL DE GMBH) 27 May 1999 (1999-05-27)	1-7,9-21
A	the whole document --- -/--	8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2000	22.02.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Meyer, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 00/23290

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	UHLMANN E ET AL: "ANTISENSE OLIGONUCLEOTIDES: A NEW THERAPEUTIC PRINCIPLE" CHEMICAL REVIEWS,US,AMERICAN CHEMICAL SOCIETY. EASTON, vol. 90, no. 4, 1 June 1990 (1990-06-01), pages 543-584, XP000141412 ISSN: 0009-2665 the whole document ---	1-21
A	COOK P. D.: "Antisensce Research and Application" 1993 , CRC PRESS INC. XP000943957 page 166 ---	1-21
A	WO 98 49348 A (ECKER DAVID J ;MONIA BRETT P (US); BAKER BRENDA F (US); BENNETT C) 5 November 1998 (1998-11-05) the whole document ---	1-21
A	CROOKE S T: "PROGRESS IN ANTISENSE THERAPEUTICS" HEMATOLOGIC PATHOLOGY,US,DEKKER, NEW YORK, NY, vol. 9, no. 2, 1995, pages 59-72, XP000651301 ISSN: 0886-0238 the whole document ---	1-21
A	MARTE B M ET AL: "PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond" TIBS TRENDS IN BIOCHEMICAL SCIENCES,EN,ELSEVIER PUBLICATION, CAMBRIDGE, vol. 22, no. 9, 1 September 1997 (1997-09-01), pages 355-358, XP004088012 ISSN: 0968-0004 the whole document ---	1-21
P,X	US 5 958 773 A (COWSERT LEX M ET AL) 28 September 1999 (1999-09-28) the whole document -----	1-21

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INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US 00/23290

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-21 partially

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: partially claims 1-21

Inhibition of AKT-1 by an antisense oligonucleotide comprising SEQ ID NO. 1

2. Claims: partially claims 1-21

Inhibition of AKT-2 by an antisense oligonucleotide comprising SEQ ID NO. 2

3. Claims: partially claims 1-21

Inhibition of CHK1 by an antisense oligonucleotide comprising SEQ ID NO. 3

4. Claims: partially claims 1-21

Inhibition of CHK2 by an antisense oligonucleotide comprising SEQ ID NO. 4 or 5

5. Claims: partially claims 1-21

Inhibition of CK1E by an antisense oligonucleotide comprising SEQ ID NO. 6

6. Claims: partially claims 1-21

Inhibition of E1AF by an antisense oligonucleotide comprising SEQ ID NO. 7 or 8

7. Claims: partially claims 1-21

Inhibition of IGFR1 by an antisense oligonucleotide comprising SEQ ID NO. 9 or 10

8. Claims: partially claims 1-21

Inhibition of ILK by an antisense oligonucleotide comprising SEQ ID NO. 11

9. Claims: partially claims 1-21

Inhibition of KRAS by an antisense oligonucleotide comprising SEQ ID NO. 12 or 13

10. Claims: partially claims 1-21

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Inhibition of MMP2 by an antisense oligonucleotide comprising SEQ ID NO. 14 or 15

11. Claims: partially claims 1-21

Inhibition of MMP9 by an antisense oligonucleotide comprising SEQ ID NO. 16

12. Claims: partially claims 1-21

Inhibition of mTyr by an antisense oligonucleotide comprising SEQ ID NO. 17 or 18

13. Claims: partially claims 1-21

Inhibition of p110a by an antisense oligonucleotide comprising SEQ ID NO. 19 or 20

14. Claims: partially claims 1-21

Inhibition of p110b by an antisense oligonucleotide comprising SEQ ID NO. 21 or 22

15. Claims: partially claims 1-21

Inhibition of PDK1 by an antisense oligonucleotide comprising SEQ ID NO. 23

16. Claims: partially claims 1-21

Inhibition of UPAR by an antisense oligonucleotide comprising SEQ ID NO. 24

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 00/23290

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		WO 0036149 A	22-06-2000